From bench to bedside: Diagnosis of Gitelman's syndrome – defect of sodium-chloride cotransporter in renal tissue

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CASE PRESENTATION

Patient 1: A 16-year-old woman was admitted owing to an acute onset of both lower extremity paralysis lasting for 5 h. Her past medical history and family history were unremarkable. She denied taking herbal medicine, diuretics, or laxatives. She appeared ill-looking and her blood pressure was 110/80 mm Hg. Neurologic examination revealed impaired sensation and decreased motor power, grade 1/5 in both lower extremities, and grade 3/5 in both upper extremities. The rest of the physical examination was normal. Initial laboratory findings revealed hypokalemic metabolic alkalosis, hypomagnesemia, and hypocalciuria (Table 2).

Patient 2: A 24-year-old woman was admitted because of cramps and paralysis of four extremities for 2 h, preceded by a dry cough, fever, and myalgia, which began 1 day before admission. Her past medical history and family history were unremarkable. She denied taking herbal medicine, diuretics, or laxatives. Her blood pressure was 110/70 mm Hg. Neurologic examination revealed grade 0–1/5 motor power and impaired sensation were detected in all four extremities. Initial laboratory findings revealed hypokalemic metabolic alkalosis, hypomagnesemia, and hypocalciuria (Table 2).

The clinical presentations and initial laboratory data of the above patients are summarized in Tables 1 and 2.

DIAGNOSIS

Hypokalemic metabolic alkalosis with hypomagnesemia and hypocalciuria presenting with acute paralysis and paresthesias.

CLINICAL FOLLOW-UP

All symptoms and abnormal neurologic signs disappeared after correction of hypokalemia and hypomagnesemia in both patients. Initially, potassium and magnesium were administered intravenously and was switched to per oral after improvement of neurologic signs. Symptoms suggesting upper respiratory viral infection in Patient 2 improved after symptomatic supportive treatment. We performed renal clearance study, gene analysis, and immunohistochemistry of sodium–chloride cotransporter (NCC) in renal tissues to elucidate the pathophysiologic cause. Informed consents were obtained from both patients, and the study protocol was approved by the Institutional Review Board of Seoul National University Hospital.

As described elsewhere,[1,2] renal clearance study using diuretics was carried out. After overnight fasting, the patients drank water (20 ml/kg of body weight) over 20 min and received intravenous infusion of 0.45% saline at a rate of 40 ml/h. Urine output was measured every 20 min followed by additional water intake (urine volume plus 20 ml/20 min). When urine flow reached a maximal level, samples of blood and urine were obtained to calculate basal values of free water clearance, chloride clearance, and distal fractional chloride reabsorption. For thiazide loading test, 100 mg of hydrochlorothiazide was orally administered to the patients. Water loading was carried out as described above. For furosemide loading test, 40 mg of furosemide was given intravenously to the patients. In both tests, clearance data were obtained when urine flow was maximal. Distal fractional chloride reabsorption was calculated using the following formula: $CH_2O/(CH_2O + CCl)$.

For the *SLC12A3* gene analysis, both RNA and genomic DNA (gDNA) were isolated from peripheral blood cells. RNA were reversely transcribed (RT) to cDNA, and four

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Table 1 | Clinical presentation of the patients

	Patient 1	Patient 2
Age (years)	16	24
Gender	Female	Female
Chief complaint	Lower extremities paralysis	Cramp and four extremities paralysis
Blood pressure (mm Hg)	110/80	110/70
Motor power	Grade 1/5 in both lower extremities and 3/5 in both upper extremities	Grade 0–1/5 in all four extremities

Table 2 | Laboratory data of the patients

	Patient 1	Patient 2	Reference values
Serum electrolytes			
Na^+ (mmol/l)	135	135	135–145
K^+ (mmol/l)	2.8	1.9	3.5–5.5
Cl^- (mmol/l)	93	94	98–110
Mg^{2+} (mmol/l)	0.7	0.55	0.75–1.25
HCO_3^- (mmol/l)	30	30.4	21–29
Ca^{2+} (mmol/l)	2.67	2.45	2.2–2.62
Plasma renin activity (ng/ml/h)	32.8	14.7	1–2.5
Serum aldosterone level (pg/ml)	403	147	50–194
Plasma pH	7.42	7.54	7.38–7.46
Urine Ca^{2+} (mmol/day)	0.2	0.35	1.75-4.49
Urine Ca^{2+}/Cr	0.008	0.013	

overlapping cDNA fragments covering most of the coding sequences of the *SLC12A3* gene were amplified by nested polymerase chain reactions and directly sequenced. Mutations detected by the nested reverse transcription-polymerase chain reaction were also confirmed by direct sequencing of the polymerase chain reaction product of the corresponding exon from gDNA.

Renal biopsy tissues from both patients were examined by immunohistochemistry for thiazide-sensitive NCC and were compared with those from the patient with nonspecific chronic tubulointerstitial nephritis as control. The renal tissues from the patients and control were fixed, dehydrated and dewaxed, and treated with methanolic H_2O_2. After treatment for permeabilization, the sections were incubated overnight at 4°C with rabbit polyclonal antibodies to the human NCC,[3] type 2 sodium–potassium–chloride cotransporter (NKCC2).[4] Thereafter, the sections were rinsed with phosphate-buffered saline and incubated in biotinylated goat anti-rabbit immunoglobulin G (BA-1000; Vector Laboratory, Burlingame, CA, USA) for 30 min at room temperature. Hematoxylin staining was used for counterstaining.

In renal clearance study, chloride clearance was markedly increased and distal fractional chloride reabsorption was remarkably decreased after furosemide loading in both patients (Table 3). However, these two parameters of renal clearance were not significantly affected by hydrochlorothiazide administration. These different responses to thiazide and furosemide are compatible with the functional diagnosis of Gitelman's syndrome.[1,2]

Table 3 | Results of the renal clearance study using furosemide and hydrochlorothiazide

	Patient 1	Patient 2
Cl^- clearance (ml/min)		
Basal	0.9	2.3
Furosemide loading	29.0	11.6
Thiazide loading	2.1	1.9
Distal fractional Cl^- reabsorption (%)		
Basal	86.1	65.0
Furosemide loading	9.7	14.0
Thiazide loading	81.4	68.0

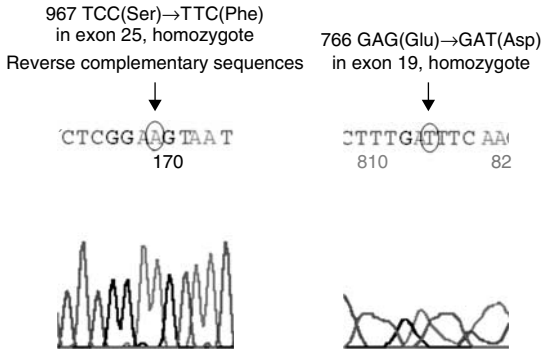

Figure 1 | Gene analysis result of Patient 1. The patient had homozygous mutation at 967 TCC codon in exon 25, which converts serine to phenylalanine (S967F).

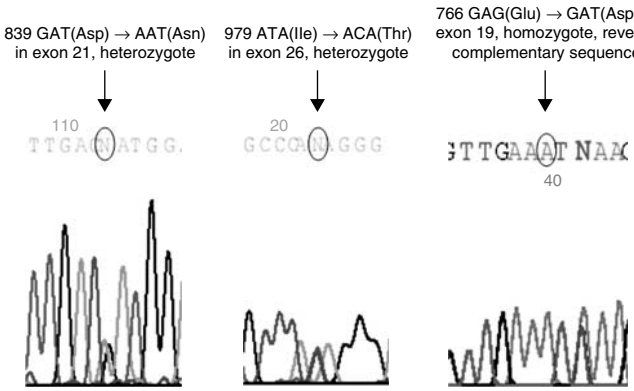

Figure 2 | Gene analysis result of Patient 2. The patient had compound heterozygous mutations at 839 GAT codon in exon 21 and 979 ATA codon in exon 26, which convert aspartate to asparagine and isoleucine to threonine, respectively (D839N/I979T).

Gene analysis revealed homozygous mutation at 967 TCC codon in exon 25, which converts serine to phenylalanine (S967F) in Patient 1 (Figure 1). In Patient 2, compound heterozygous mutations at 839 GAT codon in exon 21 and 979 ATA codon in exon 26, which convert aspartate to asparagine and isoleucine to threonine, respectively (D839N/I979T), were found (Figure 2).

In control, NCC immunohistochemistry showed distinct labeling along the apical membrane of the distal convoluted

tubule. However, renal tissues from Patient 1 were devoid of intact NCC immunostaining in the distal convoluted tubule. NCC immunostaining was absent in the apical membrane of the distal convoluted tubule and only faint staining for NCC immunoreactivity was observed in the cytoplasm of the distal convoluted tubule cells (Figure 3c). No discernible NCC immunostaining was present in the apical membrane of the distal convoluted tubule in Patient 2 (Figure 3e). In contrast, NKCC2 immunostaining was all preserved in the thick ascending limb cells in both control and the patients (Figure 3).

DISCUSSION

Two adult patients with Gitelman's syndrome were described above. Gitelman's syndrome is an autosomal-recessive renal tubular disorder characterized by hypokalemic metabolic alkalosis, hypomagnesemia, and hypocalciuria (Figure 4).[5]

It is caused by inactivating mutations in the gene for the NCC (thiazide-sensitive NCC) of the distal convoluted tubule, called *SLC12A3*.[6,7] Gitelman's syndrome should be suspected in patients with hypokalemia, metabolic alkalosis, and hypocalciuria. Patients with Gitelman's syndrome usually are normotensive, which differentiate them from patients with Liddle's syndrome. Hypomagnesemia is an important feature of typical Gitelman's syndrome, but may be absent in some patients. Therefore, hypomagnesemia is not crucial for the diagnosis of Gitelman's syndrome. Patients with Gitelman's syndrome present in adolescence or early adulthood, which differs from the earlier onset of Bartter's syndrome. Most patients with Gitelman's syndrome are asymptomatic or complain of mild intermittent cramps, fatigue, muscle weakness, or irritability. However, severe symptoms such as tetany and paralysis have been reported[8] and phenotypic variations are not uncommon in Gitelman's syndrome.[9] These phenotypic variations and the absence of standard diagnostic method make the definite diagnosis of Gitelman's syndrome more difficult.

Our two patients complained of severe paralysis or cramp, which is more suggestive of Bartter's syndrome, although their onset time and laboratory examination results were compatible with Gitelman's syndrome. Before renal biopsy, diagnosis of Gitelman's syndrome was based on clinical features and supplemented by the renal clearance study using diuretics and genotyping of *SLC12A3* in both patients. We performed immunohistochemistry of the renal tissues from both Gitelman's syndrome patients to investigate the expression of intact NCC. Similar clinical features, including laboratory abnormalities and results of the renal clearance study, can be found in chronic abuse of diuretics, surreptitious vomiting, and atypical Bartter's syndrome. Mutations of *SLC12A3*, which is responsible for Gitelman's syndrome, did not always result in functional defect of NCC and typical symptoms, although many mutations and polymorphisms were found in patients with Gitelman's syndrome.[10–12] We tried to investigate whether there is a correlation between the defect of NCC protein expression in the renal tissues and the mutations of *SLC12A3* in symptomatic Gitelman's syndrome patients.

Gitelman's syndrome is linked to inactivating mutations in the *SLC12A3* gene, which encodes the NCC. To date, more than 100 different mutations have been identified and when mutant genes were introduced into *Xenopus* oocytes, loss of NCC function, abnormal intracellular processing, and absence of NCC from the cell membrane were all demonstrated.[13] Previous *in vitro* studies have shown that most of point mutations lead to complete loss of expression and cotransporter activity, whereas some of the missense mutations in *SLC12A3* exhibit partial function.[14,15] In this study, we demonstrated that in Gitelman's syndrome, mutations in the *SLC12A3* gene are associated with defect of the intact NCC expression on the apical membrane of distal convoluted tubule. This finding seems to be relevant with some molecular defects of the NCC in Gitelman's syndrome patients and suggests a correlation between clinical functional defect and protein expression in the tissue level.

Our two patients had different mutation types: Patient 1 exhibited a classical homozygous pattern and Patient 2 revealed a compound heterozygous pattern. According to a previous report,[16] only 18% of the cases of Gitelman's syndrome exhibited homozygous mutations and about 45% of all patients were compound heterozygotes. The second most common type of mutation was from the heterozygous patients. These differences in mutation may produce phenotypic variations and may account for the variable NCC protein expression in this immunohistochemical study. Specifically, the absence of NCC immunoreactivity was complete in the patient with compound heterozygous mutation (Patient 2). Interestingly, the patient with homozygous mutation (Patient 1) was devoid of intact NCC immunostaining along the apical membrane of the distal convoluted tubule, but showed a weak cytoplasmic immunoreactivity for NCC.

It has been proposed that NCC mutations can reduce or abolish the transporter activity by five mechanisms: (1) impairment of protein synthesis, (2) impairment of protein processing, (3) interference with insertion of an otherwise functional protein into plasma membrane, (4) modification of functional properties of the cotransporter, and (5) accelerated protein removal or degradation.[17] Thus, it is conceivable that complete absence of NCC immunostaining seen in Patient 2 might be caused by impaired cotransporter protein synthesis or accelerated protein degradation.

On the other hand, Patient 1 revealed a faint NCC immunostaining, which was confined within the cytoplasm, suggesting that impairment of cotransporter protein processing or insertion into apical plasma membrane might have a role. This finding indicates that chaperone treatment might be applied to the patients with Gitelman's syndrome in the future.[18] Previously, it has been demonstrated that glycosylation is essential for efficient function and surface expression of the NCC protein[19] and that several missense mutations along the NCC were functionally inactive because of its

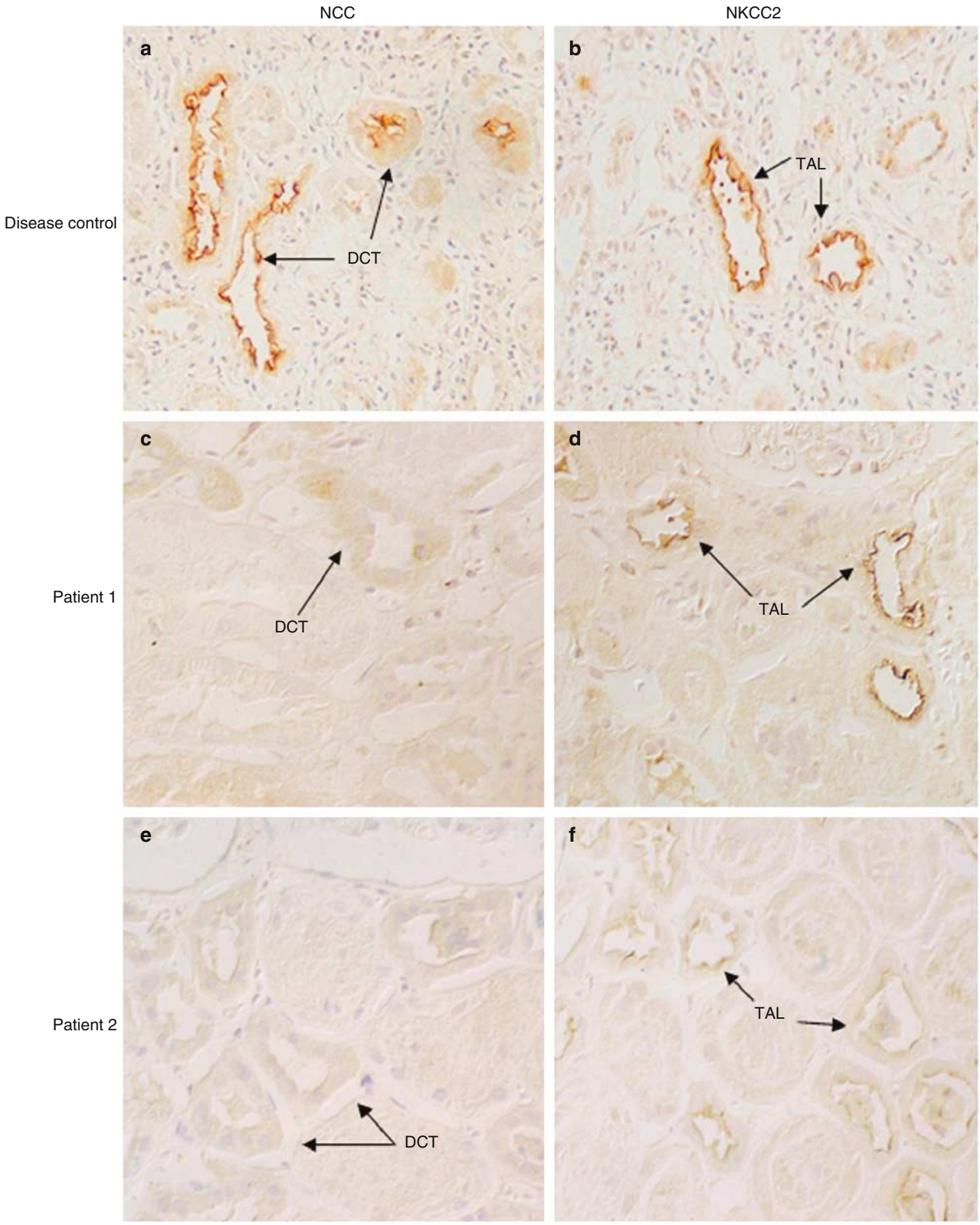

Figure 3 | Immunohistochemistry of thiazide-sensitive NCC and NKCC2 in renal biopsy tissues from two patients with (c–f) Gitelman's syndrome and the (a and b) control. Whereas the NCC immunohistochemistry in the control showed a distinct labeling along the apical membrane of the (**a**) DCT, the results of the renal tissues from the patients with Gitelman's syndrome were devoid of (**c** and **e**, see the text for details.) intact NCC immunostaining. In contrast, immunostaining for NKCC2 in the cortical TAL was all intact in both the control and patients. DCT, distal convoluted tubule; TAL, thick ascending limb.

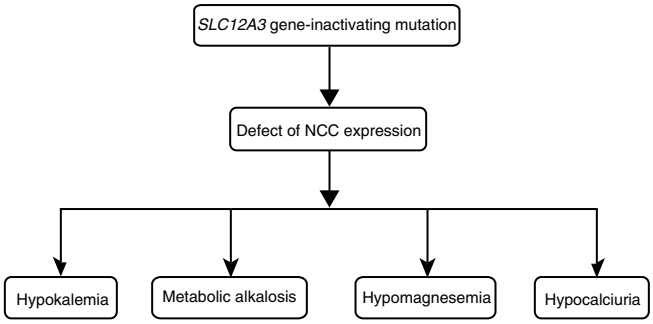

Figure 4 | Simplified pathophysiology of Gitelman's syndrome. Defect of NCC expression caused by *SLC12A3* gene-inactivating mutation results in hypokalemic metabolic alkalosis, hypomagnesemia, and hypocalciuria.

defective glycosylation.[15] Further studies are required to answer the question whether interference with NCC glycosylation or insertion into plasma membrane has a pathogenic role in some patients with Gitelman's syndrome.

The renal clearance study and genotyping, which were used for diagnosis of Gitelman's syndrome in our patients, are the two methods available for differential diagnosis. However, they have some limitations to be a standard diagnostic method. The renal clearance study is very inconvenient and cumbersome. It may show confusing results in patients with chronic abuse of diuretics, surreptitious vomiting, or atypical Bartter's syndrome, although the responses to thiazide and furosemide differ between Gitelman's syndrome and Bartter's syndrome.[1,2] It is also limited in patients with azotemia, although renal function is nearly always preserved in Gitelman's syndrome.[8] Genotyping is known to be the best method for confirming the diagnosis, but it is impractical owing to laboratory unavailability in most hospitals.[10–12] A vast number of different novel mutations and polymorphisms of *SLC12A3* in Gitelman's syndrome in addition to the impracticability makes genotyping difficult on a routine basis. Immunohistochemistry of NCC in the renal tissues of patients may be a reliable diagnostic method, but it is invasive and impractical in most hospitals. Therefore, a simple, reliable diagnostic method capable of detecting functional defect of NCC is needed. It has also been shown that sodium transporters such as type 3 sodium–hydrogen exchanger, NKCC2, and NCC could be detected in urine by immunoblotting,[20] which may have some potential in the diagnosis of Gitelman's syndrome.

CLINICAL PERSPECTIVE AND CONCLUSIONS

Two patients with Gitelman's syndrome who were described above presented with severe symptoms, which are more common in Bartter's syndrome. However, their laboratory features and clearance studies were compatible with Gitelman's syndrome. We confirmed their mutations of *SLC12A3* and the defect of NCC expression in their renal tissues. It is the first report that directly showed the defect of intact NCC in the renal tissues of Gitelman's syndrome patients. Although immunohistochemistry of NCC is a method to ascertain the defect of NCC expression, a new diagnostic method is needed owing to its invasiveness and impracticability. Further study is required to develop a new diagnostic method, which can correlate functional defect with gene defect.

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